

Chemometric Analysis of Chromatographic Data in Stability Investigation of Cephalosporins

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The specific aim of this investigation was to study the kinetics of the degradation of cefazolin, cefaclor, cefuroxime axetil, and cefepime in aqueous solution, in the presence (or absence) of various redox agents (iodine solution, potassium permanganate, hydrogen peroxide, sodium thiosulfate, and ascorbic acid) as a function of temperature. Various factors, such as concentration of the analyzed compounds and redox agents, storage time, and temperature, were analyzed. The degradation process of chosen antibiotics was observed chromatographically and fitted to the kinetic models, obtaining model parameters (k , $t_{0.1}$, $t_{0.5}$). Principal component analysis (PCA), parallel factor analysis (PARAFAC), and hierarchical cluster analysis (HCA) methods were carried out to interpret the dependencies between these factors on the drug stability.

Keywords: Cephalosporins, redox agents, degradation process, chemometrics

Introduction

In the middle of the twentieth century, the discovery of penicillin fundamentally changed the treatment of patients with infectious diseases. β -Lactam antibiotics, including penicillin derivatives, cephalosporins, monobactams, carbapenems, and β -lactamase inhibitors, are one of the most frequently used antimicrobial agents [1]. These semisynthetic antibiotics differ in the pharmacokinetic properties and antibacterial spectrum according to the presence of a substituent attached to the cephem ring.

In the presented paper, we focus on four cephalosporins belonging to various generations. Cefazolin (first-generation cephalosporin) is a semisynthetic penicillin derivative with narrow spectrum of its activity, covering some gram-positive and a few gram-negative aerobic bacteria. It is a chlorinated derivative of cephalexin, a recently introduced oral cephalosporin antibiotic, designed for the treatment of urinary tract infection, otitis media, skin infections, and respiratory infections.

The next one, cefaclor (second-generation cephalosporin) differs structurally from all investigated substances. The negative inductive effect of chlorine substituent attracts electrons more strongly and forms H-bonding with polar solvents.

Cefuroxime axetil is a second-generation cephalosporin used to treat or to prevent infections proven (or strongly suspected to be caused by) bacteria. It has a carbamoyl group which gives a sizable metabolic stability, a methoxyimino group which causes its high affinity to β -lactamase, and 1-acetoxyethyl ester group, providing its lipophilicity and promoting the intestinal absorption.

The last one, cefepime, is a fourth-generation cephalosporin with a broad spectrum of activity against many gram-positive and gram-negative bacteria. It contains one carboxylic group, one aminothiazole ring, and one positively charged quaternary nitrogen, responsible for the formation of zwitterionic species over a broad pH range.

Because cephalosporins are relatively safe and have a broad spectrum, they are the most commonly prescribed class of all an-

tibiotics. Analytical methods for their quality control are interesting not only from the view of pharmaceutical analysis but also touch food safety and environmental protection (for example, monitoring antibiotic residues in milk, edible tissues of animals [2], and wastewater from butcheries or hospitals [3]). Direct separation of the particular cephalosporins is difficult because they demonstrate small differences in polarity and lack chromophore or fluorophore [4]. Among the separation techniques, high-performance liquid chromatography (HPLC) has been widely used for the analysis of cephalosporins [5–9]. Thin-layer chromatography (TLC) has also been a popular technique [10–15] due to its simplicity, the ability for simultaneous analysis of large number of samples, a large choice of various stationary and mobile phases, and the possibility of two-dimensional separation.

The ongoing emergence of antibiotic-resistant bacterial pathogens forces us to improve our knowledge on the mechanisms of drug action, with the goal of enhancing the effectiveness of existing antibiotics. Many antibiotics owe their lethal effects, at least in part, to oxidative stress and to the damage incurred as a result of the antibiotic-induced generation of reactive oxygen species [16–19]. Although this model is still controversial [20, 21], some researchers have proposed that enhancing the production of endogenous oxidants in order to potentiate the activities of known antibiotics may be a practicable therapeutic strategy to extend antibiotic arsenal [22]. Therapy with broad spectrum cephalosporins is an important risk factor for hospital acquired enterococcal infections. Djoric et al. searched the genetic determinants of cephalosporin resistance in *Enterococci faecalis* and discovered that oxidative stress drives enhanced intrinsic resistance to antibiotics. Hydrogen peroxide exposure appears to enhance cephalosporin resistance by triggering the specific activating pathway to promote cephalosporin resistance [23]. Previous studies indicated that ceftriaxone has antioxidant role in the brain and nervous system [24] and benefits cyclosporine A (Cyc-A)-induced oxidative stress in kidney of animals by creating antioxidant and oxidative system [25]. Renal damage is ameliorated by administration of

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CycA. Ceftriaxone reduces CycA and thereby increases the therapeutic index of this drug by reducing toxicity that may occur through free radical mechanism [26].

Moreover, pharmaceuticals are especially sensitive to environmental factors. There is a need to define strict storage conditions to maintain product integrity and activity [27]. Stability testing of the active substance of finished product delivers evidence how the quality of a drug varies with time and if it is influenced by various factors, such as temperature, humidity, light, and oxidation. The knowledge obtained from stability studies provides information about potential products and possible degradation pathways of the drug, as well as about the interactions between the drug and the excipients. The results are useful in developing of manufacturing processes (packaging, storage, product's shelf life, and expiration dates) [28, 29].

Chemometric is a branch of science that derives data by the application of mathematical and statistical models. The multivariate statistics are used for data collection, process modeling, pattern recognition and classification, signal correction and compression, and statistical process control. The chemometric techniques have advantage in the quality assurance and quality control of pharmaceutical solid dosage forms, in the evaluation of properties of pharmaceutical powders and tablet [30–32]. Principal component analysis (PCA) is a simple method for receipting relevant information from multivariate data sets, identifying trends and clusters in data, and expressing the data to highlight both similarities and differences between objects [33–35]. It can be used to find a correlation structure of variables [36, 37] and for reducing the number of variables during the monitoring of a process [38–40]. The first axis depicts a linear combination of original variables, explaining maximum possible total variance, the second one — a combination representing maximal remaining variance (but orthogonal to the first combination), and so on [41, 42]. Capone et al. applied multivariate statistical methods (PCA and hierarchical cluster analysis [HCA]) to find key features involving molecules, descriptors, and anticancer activity [43]. Mehl et al. used hyperspectral image analysis (HIS) and PCA, to reduce the information resulting from HIS and to identify three spectral bands capable of separating normal from contaminated apples [44]. Parallel factor analysis (PARAFAC) is a decomposition method for modeling three-way or higher-way data [45–48]. It is a method of decomposing multi-dimensional arrays in order to focus on the features of interest and ensures a distinct illustration of the results. PARAFAC is utilized to decompose the multi-way data into a linear combination of score matrices [49]. Its basic idea is very flexible and general, which allows application in many chemical problems [50–53]. HCA is a computerized tool for large data set analysis, one of the most promising techniques for rapid examination of various biological and medical data sets. It is an approach to find discrete groups with varying degrees of similarity or dissimilarity in a data set represented by a similarity or dissimilarity matrix. These groups are hierarchically organized as the algorithm proceeds, which results in a graph called dendrogram [54, 55].

Under certain conditions, hydrogen peroxide might influence resistance to antibiotics with diverse cellular targets. Therefore, our team investigated the stress influence on cephalosporin antibiotics, using stress agents such as hydrogen peroxide at different concentrations (0.1 and 0.3%), sodium thiosulfate, potassium permanganate, iodine solution, and ascorbic acid. In previous researches, we elaborated also simple and rapid TLC methods for the determination of selected cephalosporins from various generations. In this paper, we applied those methods for the study of cefazolin (first-generation cephalosporin), cefuroxime axetil (second generation), cefaclor (second generation), and cefepime (fourth generation) after the reaction between any of a drugs

with a redox agents at various temperatures (22 and 36 °C). The current research was designed to evaluate the chemical stability of the antibiotics and to describe the degradation in terms of the kinetic parameters acquired by thin-layer chromatographic assay and chemometric tools for comparative analysis of degradation profiles of the drugs.

Experimental

Chemical Reagents. Standard substances of cefaclor QA198A, LOT Y004873 (Eli Lilly, Italia), cefuroxime axetil (amorphous) 2CRA00798A (Ranbaxy, India), cefazolin sodium USP L0C345 (Biochemie GmbH, Kundl), and cefepime hydrochloride No: H0G278 USP (Rockville, USA) were used.

The following substances and solvents were of analytical grade: potassium permanganate, hydrogen peroxide, iodine, sodium thiosulfate and ascorbic acid ((5*R*)-5-[(1*S*)-1,2-dihydroxyethyl]-3,4-dihydroxy-2(5*H*)-furanone), ethyl acetate, ethanol, chloroform, 2-propanol purchased from POCh (Gliwice, Poland), and glacial acetic acid (>99.85%) from (Sigma-Aldrich, Germany) were applied.

Standard Solutions for Analysis. For the determination, the solutions of 0.2% v/v concentration were prepared by dissolving the standard substance in the mixture of water and methanol (1:1 v/v).

Redox Agent Solutions. The proper amounts of the redox agents were dissolved with distilled water in 50.0 mL volume flask. The solutions at a concentration of 0.1 and 0.3% v/v of hydrogen peroxide, 0.01 mol/L of potassium permanganate, 0.05 mol/L of iodine solution, 0.10 mol/L of sodium thiosulfate, and 0.10 mol/L of ascorbic acid were applied for further analysis.

Testing Solutions. To 5.0 mL glass ampoules, 1.0 mL of 0.2% v/v standard solution of appropriate cephalosporin, followed by 0.5–2.5 mL of redox agent solution, were added and then shaken. The testing solutions were then heated at 22 °C or 36 °C within a certain period of time. Then, the solutions were applied on TLC F₂₅₄ plates in a volume of 15 µL. The assay was carried out three times for each sample, taking the mean value as a result.

Chromatography. The studies were carried out using previously validated TLC–densitometric methods [12, 15]. On 17 × 10 cm Silica Gel TLC F₂₅₄ chromatographic plates (Merck, Germany, No. 1.05554), 15 µL of the relevant solutions were applied as 10 mm bands (8 mm apart from the edge) by use of a sample applicator Linomat V (Camag, Muttenz, Switzerland), equipped with a 100 µL syringe. The constant application rate of 250 nL/s was used. The plates were then developed over a distance of 95 mm in a vertical chamber (size 18 × 9 × 18 cm, Sigma-Aldrich), previously saturated with an appropriate mobile phase for 10 min at room temperature. The mixtures consisted of ethanol–2-propanol–glacial acetic acid–water (4:4:1:3 v/v/v/v) for cefepime estimation and chloroform–ethyl acetate–glacial acetic acid–water (4:4:4:1 v/v/v/v) for cefaclor, cefazolin, and cefuroxime axetil were used as the mobile phases. The chromatograms were dried at room temperature before densitometric analysis with TLC Scanner 3 equipped with Cats 4 software (Camag, Muttenz, Switzerland). The reflectance/absorbance mode within the range of 200 to 400 nm was chosen. The analytical wavelengths for particular cephalosporins were chosen for further studies after preliminary experiments (280 nm for cefazolin, 274 nm for cefaclor, 285 nm for cefuroxime axetil, and 266 nm for cefepime). The slit dimension was 8 × 0.60 mm, and the scanning speed was set to 20 mm/s.

The degradation process was characterized by the decreasing concentration of analyzed cephalosporin. For quantitative determination, the scan areas of appropriate peaks were recorded,

and the percentage concentrations of each constituent were computed. The obtained results are reported as the mean value of three measurements.

Specificity. Specificity of the method was assessed by comparison of the chromatograms obtained from pure standard substances with chromatograms obtained from cephalosporin solutions after the incubation, and also with a blank chromatogram. Peak areas of the analyzed substances, their shape, the purity of the peaks, and the retardation factor (R_F) values were taken into account. The peak purity was estimated by comparing the spectra at three points across each peak, that is, peak start, peak apex, and peak end.

Kinetic Testing. The degradation process of cephalosporins was studied by fitting the appropriate kinetic parameters [56]. The order of the reaction was estimated, and the reaction rate constants (k), the half-life ($t_{0.5}$), and the time ($t_{0.1}$) at which the concentration of determined compound is reduced by 10% were also calculated. The parameters were designated using the following formulas: $k = 2.303(\log c_1 - \log c_2) / (t_2 - t_1)$, $t_{0.5} = 0.693/k$, and $t_{0.1} = 0.1053/k$.

Software Tools. The obtained data were analyzed by commercially available statistic software packages: Statistica v10 (StatSoft, USA), GNU R 2.15.1 (www.r-project.org, PCA analysis), and Matlab R2013a (MathWorks, USA) with N-way toolbox (PARAFAC). To evaluate the linearity of the kinetics, a regression analysis was carried out by determining following parameters: the correlation coefficient (r) and the standard error of the slope, intercept, and estimate (S_a , S_b , S_e).

Results and Discussion

The changes of cefazolin, cefaclor, cefuroxime axetil, and cefepime concentration under various stress conditions were registered in the current research using earlier elaborated TLC methods with densitometric detection [12, 15]. The separation of antibiotics and their degradation products was achieved with mobile phase containing ethanol–2-propanol–glacial acetic acid–water (4:4:1:3 v/v/v/v) for cefepime (retardation factor R_F was about 0.21), while mixture of chloroform–ethyl acetate–glacial acetic acid–water (4:4:4:1 v/v/v/v) was used for cefaclor ($R_F = 0.23$), cefazolin ($R_F = 0.30$), and cefuroxime axetil ($R_F = 0.94$).

The analysis of samples (exposed to different redox agents and temperature) indicated the appearance of several new peaks; however, there were no signs of interference with the individual peaks. Taking into consideration the chromatographic separation of these degradation products from the main drug and the similarity of the ultraviolet (UV) spectra (in the range of 200–400 nm) for the freshly prepared and the degraded samples of each antibiotic, it was concluded that those analytical methods can be considered as stable. A precipitate was not formed in any analyzed sample. It was not possible to perform the planned stability testing for cefazolin in the presence of ascorbic acid and iodine, and for cefaclor with ascorbic acid. In this case, the registered peaks for active substances and redox agents demonstrated to have too similar retardation factors and maxima of the absorption spectra (Figure 1).

In the next step of our studies, the influence of redox agent and its concentration on the degradation of cephalosporins were analyzed. With increase of redox agents concentration, the quantity of active substances decreased. When the analyzed solutions contained redox agents in volume 0.25–1.00 mL, the presence of a single peak derived from active substance was revealed. At higher concentrations, the additional peaks appeared and the separation worsened. Finally, studies of the degradation process of cephalosporins were continued with the constant ratio of each cephalosporin and each redox agent (1:1 v/v). A good specificity of the assay was obtained in these conditions. It was then found that the time of interaction between a drug and each agent has also an influence on the concentrations of individual degradants, while their peak positions in the chromatograms remained unchanged. Initial (immediately after preparation) concentration in the samples was defined as 100.00%. Subsequent concentrations were expressed as a percentage of the starting concentration for 3 replicate of the samples, determined in specified period of time and temperature. Starting concentration (at the time “0”) and the percent remaining on each day, for the all samples included in the study plan, at temperature 22 and 36 °C are reported in Table 1.

Among all analyzed cephalosporins, only cefazolin was chemically stable during our experiments; about 100.00% concentration remained in the solutions with all tested redox agents. It could be observed that the registered peaks decreased, which indicates that the drugs underwent degradation. For each antibiotic (cefazolin, cefaclor, cefuroxime axetil, and cefepime),

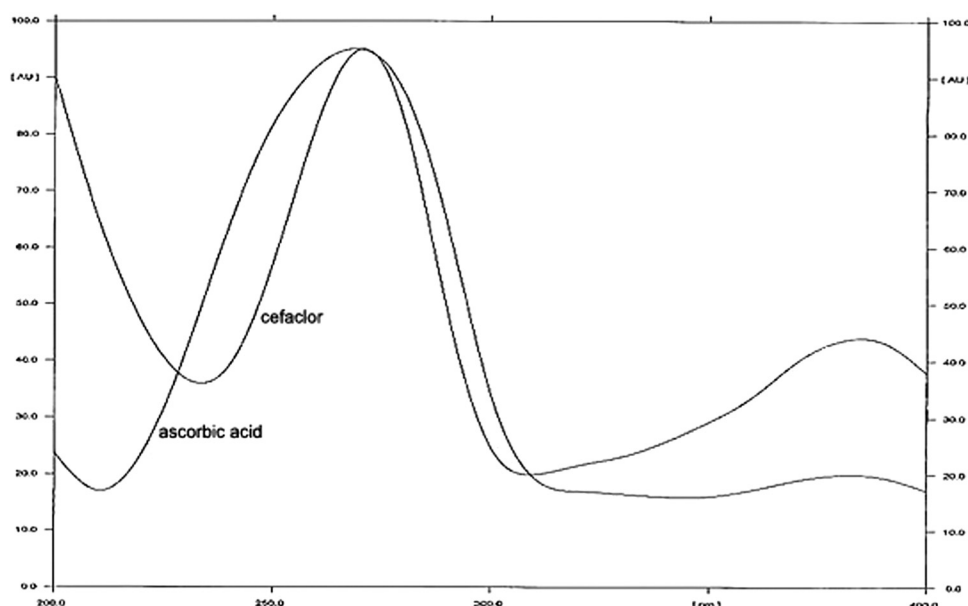


Figure 1. Absorption spectra registered for cefaclor and ascorbic acid

Table 1. The results for the determination of cephalosporins (%) after incubation at 22 and 36 °C with various redox agents

Agent/Time [day]	0	1	7	14	21	28	35
Cefazolin							
22 °C							
Water	100.00	99.02	90.68	86.31	81.09	79.92	71.76
0.1% Hydrogen peroxide	100.00	91.74	97.80	81.68	78.55	72.50	69.63
0.3% Hydrogen peroxide	100.00	90.27	80.49	73.87	67.20	62.10	50.24
Potassium permanganate	100.00	97.81	85.63	65.56	42.60	21.90	0.00
Thiosulfate sodium	100.00	92.06	82.38	76.56	65.65	64.09	56.32
36 °C							
Water	100.00	92.36	81.88	72.19	63.41	55.10	40.50
0.1% Hydrogen peroxide	100.00	90.12	68.54	51.58	28.63	8.45	0.00
0.3% Hydrogen peroxide	100.00	78.79	53.26	24.34	0.00	0.00	0.00
Potassium permanganate	100.00	87.20	63.61	44.10	22.04	0.00	0.00
Thiosulfate sodium	100.00	83.61	57.43	43.48	19.22	0.00	0.00
Cefaclor							
22 °C							
Water	100.00	97.62	95.36	92.49	88.18	85.01	33.46
0.1% Hydrogen peroxide	86.93	84.61	81.36	78.21	76.01	71.39	68.01
0.3% Hydrogen peroxide	83.99	81.12	77.32	74.25	70.35	65.79	62.16
Potassium permanganate	93.98	86.67	82.24	75.82	65.07	52.11	45.20
Iodine solution	100.00	94.40	90.50	82.40	77.79	73.16	67.72
Thiosulfate sodium	100.00	84.47	71.52	40.76	22.27	0.00	0.00
36 °C							
Water	100.00	92.60	79.88	69.40	54.16	42.20	38.21
0.1% Hydrogen peroxide	87.87	72.55	59.74	46.33	30.04	16.12	0.00
0.3% Hydrogen peroxide	82.10	68.70	55.00	30.17	13.29	0.00	0.00
Potassium permanganate	95.40	87.43	70.96	57.38	36.66	21.49	13.61
Iodine solution	100.00	90.22	73.41	51.99	29.48	16.42	5.92
Thiosulfate sodium	100.00	80.91	34.67	0.00	0.00	0.00	0.00
Cefuroxime axetil							
22 °C							
Water	100.00	96.00	87.62	76.32	68.20	61.08	53.07
0.1% Hydrogen peroxide	64.88	60.35	52.55	38.07	28.21	23.36	12.45
0.3% Hydrogen peroxide	55.57	49.07	40.65	29.73	19.34	10.62	3.51
Potassium permanganate	58.25	51.12	45.14	33.58	19.23	11.47	3.86
Iodine solution	89.38	83.00	70.71	61.78	52.18	43.93	31.45
Thiosulfate sodium	100.00	78.32	63.71	49.40	24.66	7.33	0.00
Ascorbic acid	79.75	76.56	68.83	47.26	27.02	14.29	0.00
36 °C							
Water	100.00	88.67	64.59	44.01	23.97	9.46	0.00
0.1% Hydrogen peroxide	61.57	47.42	24.31	0.00	0.00	0.00	0.00
0.3% Hydrogen peroxide	50.29	38.40	0.00	0.00	0.00	0.00	0.00
Potassium permanganate	60.44	46.08	24.30	0.00	0.00	0.00	0.00
Iodine solution	86.99	75.25	53.70	36.78	14.79	0.00	0.00
Thiosulfate sodium	100.00	86.49	63.08	38.70	14.58	0.00	0.00
Ascorbic acid	81.01	67.46	41.88	18.60	0.00	0.00	0.00
Cefepime							
22 °C							
Water	100.00	88.65	78.43	66.81	47.32	35.63	27.43
0.1% Hydrogen peroxide	87.40	74.10	69.20	50.15	35.98	21.90	5.60
0.3% Hydrogen peroxide	66.08	57.88	43.15	25.06	3.22	0.00	0.00
Potassium permanganate	75.59	66.15	55.59	43.15	22.58	12.80	0.00
Iodine solution	89.99	76.48	64.50	49.30	34.17	24.81	11.90
Thiosulfate sodium	96.54	79.57	68.26	50.21	38.64	22.73	7.70
Ascorbic acid	90.86	79.44	67.28	54.15	42.89	20.57	7.14
36 °C							
Water	100.00	86.40	74.72	57.81	38.46	22.90	10.07
0.1% Hydrogen peroxide	78.98	65.56	53.44	39.65	24.07	6.99	0.00
0.3% Hydrogen peroxide	63.99	55.71	35.52	16.03	0.00	0.00	0.00
Potassium permanganate	75.03	63.16	46.44	27.23	11.09	0.00	0.00
Iodine solution	87.70	76.58	59.40	45.33	29.09	15.30	5.30
Thiosulfate sodium	90.12	74.73	64.20	49.22	30.11	17.63	0.00
Ascorbic acid	88.90	71.22	57.94	42.26	27.08	11.99	0.00

this process occurred with a different rate, such that, in the presence of iodine solution, 67.72% at 22 °C and 5.92% at 36 °C of cefaclor remained at the end of the degradation study, while, for cefuroxime axetil, only 31.45% at lower and 0.00% at higher temperature. About 91.55% of the cefazolin was reduced after 28 days of exposure to 0.1% hydrogen peroxide at 36 °C, and 0.00% remained after exposure to the other redox agents. Long-term exposure of cefepime (35 days at room temperature) caused a reduction in quantity to 5.60% (in presence of 0.1% hydrogen peroxide), 11.90% (in iodine solution), about 7% (in thiosulfate sodium and ascorbic acid solutions), and 0.00% in solutions containing 0.3% hydrogen peroxide and potassium permanganate.

The degradation processes were characterized by the reaction order and the appropriate kinetic parameters (k , $t_{0.1}$, $t_{0.5}$). The obtained correlations were linear (correlation coefficient $[r]$, higher than 0.98), and it induced that reactions of the degradation for all analyzed drugs proceed according to the first-order kinetics (Table 2).

According to this model, the rate constant (k) was in the range from $3.58 \cdot 10^{-4}/h$ for cefaclor to $6.00 \cdot 10^{-3}/h$ for cefepime, both in 0.3% v/v hydrogen peroxide at room temperature, while, in 36 °C, it was in the range from $6.31 \cdot 10^{-3}/h$ for cefaclor in presence of sodium thiosulfate to $1.12 \cdot 10^{-2}/h$ for cefuroxime axetil in 0.3% hydrogen peroxide. Considering the kinetic data, the rate constants appeared to be higher in 36 °C

Table 2. Calibration parameters for the kinetic evaluation of analyzed cephalosporins with various redox agents

Agent/Calibration parameter	<i>a</i>	<i>b</i>	<i>Sa</i>	<i>Sb</i>	<i>Se</i>	<i>r</i>
Cefazolin 22 °C						
Water	-0.7973	99.4704	0.0447	0.8767	1.4754	0.9922
0.1% Hydrogen peroxide	-0.8869	98.5594	0.0440	0.8635	1.4533	0.9939
0.3% Hydrogen peroxide	-1.3391	96.7300	0.0842	1.6532	2.7823	0.9903
Potassium permanganate	-2.8773	102.6418	0.0798	1.5666	2.6367	0.9981
Thiosulfate sodium	-1.3536	96.0771	0.0735	1.4429	2.4284	0.9927
36 °C						
Water	-1.5453	95.6062	0.0883	1.7339	2.9179	0.9919
0.1% Hydrogen peroxide	-2.8689	93.0606	0.1709	3.3556	5.6473	0.9912
0.3% Hydrogen peroxide	-4.4533	89.5763	0.4574	5.3619	8.1468	0.9845
Potassium permanganate	-3.3899	92.9276	0.1916	3.0000	4.8123	0.9937
Thiosulfate sodium	-3.3231	89.9462	0.2859	4.4771	7.1817	0.9855
Cefaclor 22 °C						
Water	-0.4679	98.8162	0.0278	0.5452	0.9176	0.9913
0.1% Hydrogen peroxide	-0.5054	85.7275	0.0254	0.5001	0.8417	0.9937
0.3% Hydrogen peroxide	-0.5879	82.4714	0.0275	0.5401	0.9091	0.9946
Potassium permanganate	-1.3352	91.8036	0.0784	1.5403	2.5922	0.9915
Iodine solution	-0.8612	96.7516	0.0611	1.1991	2.0181	0.9877
Thiosulfate sodium	-3.4108	93.5312	0.2054	3.2163	5.1591	0.9928
36 °C						
Water	-1.7739	94.9272	0.1241	2.4351	4.0981	0.9880
0.1% Hydrogen peroxide	-2.3036	79.5469	0.1416	2.7792	4.6773	0.9907
0.3% Hydrogen peroxide	-2.8445	75.2032	0.2113	3.3086	5.3072	0.9891
Potassium permanganate	-2.3498	90.2864	0.1305	2.5628	4.3130	0.9924
Iodine solution	-2.7007	93.3881	0.1719	3.3751	5.6801	0.9900
Thiosulfate sodium	-6.8961	91.8269	0.8583	6.7308	9.5958	0.9849
Cefuroxime axetil 22 °C						
Water	-1.3145	97.3778	0.0603	1.1842	1.9928	0.9948
0.1% Hydrogen peroxide	-1.4618	62.1172	0.0838	1.6437	2.7662	0.9919
0.3% Hydrogen peroxide	-1.4493	51.7303	0.0746	1.4648	2.4652	0.9934
Potassium permanganate	-1.5348	55.0484	0.0816	1.6013	2.6949	0.9930
Iodine solution	-1.5389	85.0797	0.0845	1.6586	2.7915	0.9925
Thiosulfate sodium	-2.7210	87.2717	0.2454	4.8161	8.1054	0.9801
Ascorbic acid	-2.3495	80.3931	0.0921	1.8076	3.0421	0.9962
36 °C						
Water	-2.8259	90.0318	0.2352	4.6152	7.7673	0.9831
0.1% Hydrogen peroxide	-4.1245	56.0096	0.4974	3.9009	5.5614	0.9858
0.3% Hydrogen peroxide	-6.9107	47.9919	0.6634	2.7083	3.5520	0.9954
Potassium permanganate	-4.0266	54.8515	0.4966	3.8944	5.5521	0.9851
Iodine solution	-2.9943	80.0170	0.2013	3.1530	5.0577	0.9911
Thiosulfate sodium	-3.4997	91.8875	0.2497	3.9102	6.2722	0.9900
Ascorbic acid	-3.7073	73.6726	0.3577	4.1931	6.3709	0.9863
Cefepime 22 °C						
Water	-2.0196	94.0498	0.1276	2.5038	4.2138	0.9902
0.1% Hydrogen peroxide	-2.1998	82.3498	0.1135	2.2274	3.7486	0.9934
0.3% Hydrogen peroxide	-2.3586	61.3086	0.1690	2.6468	4.2457	0.9900
Potassium permanganate	-2.0950	71.1329	0.118	1.9970	3.3609	0.9942
Iodine solution	-2.0814	81.6823	0.1495	2.9344	4.9385	0.9873
Thiosulfate sodium	-2.3184	87.0565	0.1637	3.2121	5.4509	0.9878
Ascorbic acid	-2.2475	85.7944	0.1165	2.2858	3.8469	0.9993
36 °C						
Water	-2.4670	93.1222	0.1267	2.4871	4.1858	0.9935
0.1% Hydrogen peroxide	-2.1752	71.3226	0.1467	2.8792	4.8456	0.9888
0.3% Hydrogen peroxide	-2.9760	59.8434	0.2110	2.4737	3.7585	0.9925
Potassium permanganate	-2.5932	67.8448	0.2117	3.3155	5.3183	0.9869
Iodine solution	-2.2682	79.8750	0.1517	2.9762	5.0088	0.9890
Thiosulfate sodium	-2.3812	82.6312	0.1349	2.6480	4.4564	0.9921
Ascorbic acid	-2.3486	78.3210	0.1813	3.5585	5.9987	0.9854

a - slope; *b* - intercept; *Sa* - standard error of the slope; *Sb* - standard error of the intercept; *Se* - standard error of the estimate; *r* - correlation coefficient.

compared to the room temperature, which demonstrates a lower stability of the antibiotics in an elevated temperature. The degradation rate of the analyzed cephalosporins depends both on the temperature and the presence of redox agents. Visible differences in stability of tested compounds are also confirmed by the half-life ($t_{0.5}$) and $t_{0.1}$ values (Table 3).

In the continuation of our study, chemometric tools were applied to the results to enhance their interpretation. In the beginning, principal component analysis was performed on the matrix containing 7 columns (time points) and 50 rows (degradation curves, i.e., unique tested combinations of drug, degrading agent, and temperature). PCA was done in unscaled

and centered way. It was shown that 84.61% of overall variance was located in the first principal component, whereas 11.84% was located in the second. Taking into account that 96.45% of variance is explained by two principal components, it can be concluded that this data set is easy to be compressed and almost whole information inside can be expressed as two separate, orthogonal trends. Analyzing the values of loadings (Figure 2A), one can conclude that the percentage values after 35 days are almost uncorrelated with these values right after start of degradation. The subsequent points are however inter-correlated, and the arrows of subsequent time points form some “handheld fan.” Therefore, the largest source of the

Table 3. The kinetic data of the degradation of cephalosporins in the presence of redox agents at various temperatures

Temperature/agent	Kinetic parameter	Cefazolin	Cefaclor	Cefuroxime axetil	Cefepime
22 °C					
Water	<i>k</i>	$3.95 \cdot 10^{-4}$	$2.15 \cdot 10^{-4}$	$7.54 \cdot 10^{-4}$	$1.54 \cdot 10^{-3}$
	<i>t</i> _{0.5}	1755.32	2972.09	919.10	450.00
	<i>t</i> _{0.1}	266.72	489.77	139.66	68.38
0.1% v/v Hydrogen peroxide	<i>k</i>	$4.31 \cdot 10^{-4}$	$2.92 \cdot 10^{-4}$	$1.97 \cdot 10^{-3}$	$3.27 \cdot 10^{-3}$
	<i>t</i> _{0.5}	1607.89	2373.29	351.78	211.93
	<i>t</i> _{0.1}	244.32	360.62	53.45	32.20
0.3% v/v Hydrogen peroxide	<i>k</i>	$8.20 \cdot 10^{-4}$	$3.58 \cdot 10^{-4}$	$3.29 \cdot 10^{-3}$	$6.00 \cdot 10^{-3}$
	<i>t</i> _{0.5}	845.12	1935.75	210.64	115.50
	<i>t</i> _{0.1}	128.41	294.13	32.00	17.60
Potassium permanganate	<i>k</i>	$2.26 \cdot 10^{-3}$	$8.72 \cdot 10^{-4}$	$3.23 \cdot 10^{-3}$	$2.64 \cdot 10^{-3}$
	<i>t</i> _{0.5}	306.64	794.72	214.55	262.50
	<i>t</i> _{0.1}	47.86	120.76	32.61	39.89
Thiosulfate sodium	<i>k</i>	$6.84 \cdot 10^{-4}$	$2.98 \cdot 10^{-3}$	$3.89 \cdot 10^{-3}$	$3.01 \cdot 10^{-3}$
	<i>t</i> _{0.5}	1013.16	232.55	178.15	230.23
	<i>t</i> _{0.1}	154.85	35.34	27.07	34.98
Iodine solution	<i>k</i>	NA	$4.64 \cdot 10^{-4}$	$1.24 \cdot 10^{-3}$	$2.41 \cdot 10^{-3}$
	<i>t</i> _{0.5}	NA	1493.53	558.87	287.55
	<i>t</i> _{0.1}	NA	226.94	84.91	43.69
Ascorbic acid	<i>k</i>	NA	NA	$2.56 \cdot 10^{-3}$	$3.03 \cdot 10^{-3}$
	<i>t</i> _{0.5}	NA	NA	270.70	228.71
	<i>t</i> _{0.1}	NA	NA	41.13	34.75
36 °C					
Water	<i>k</i>	$1.08 \cdot 10^{-3}$	$1.15 \cdot 10^{-3}$	$3.51 \cdot 10^{-3}$	$2.73 \cdot 10^{-3}$
	<i>t</i> _{0.5}	641.67	602.61	197.44	253.38
	<i>t</i> _{0.1}	139.17	91.57	30.00	38.57
0.1% v/v Hydrogen peroxide	<i>k</i>	$3.68 \cdot 10^{-3}$	$2.52 \cdot 10^{-3}$	$5.53 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$
	<i>t</i> _{0.5}	188.32	275.00	125.32	191.97
	<i>t</i> _{0.1}	28.61	38.71	19.04	29.17
0.3% v/v Hydrogen peroxide	<i>k</i>	$4.21 \cdot 10^{-3}$	$3.61 \cdot 10^{-3}$	$1.12 \cdot 10^{-2}$	$4.12 \cdot 10^{-3}$
	<i>t</i> _{0.5}	164.61	191.97	61.89	168.20
	<i>t</i> _{0.1}	25.01	29.17	9.40	25.56
Potassium permanganate	<i>k</i>	$3.00 \cdot 10^{-3}$	$2.32 \cdot 10^{-3}$	$5.43 \cdot 10^{-3}$	$3.79 \cdot 10^{-3}$
	<i>t</i> _{0.5}	231.00	298.71	127.62	182.85
	<i>t</i> _{0.1}	35.10	45.39	19.39	27.78
Thiosulfate sodium	<i>k</i>	$3.28 \cdot 10^{-3}$	$6.31 \cdot 10^{-3}$	$3.82 \cdot 10^{-3}$	$2.43 \cdot 10^{-3}$
	<i>t</i> _{0.5}	211.28	109.83	181.41	285.19
	<i>t</i> _{0.1}	32.10	16.69	27.57	43.33
Iodine solution	<i>k</i>	NA	$3.37 \cdot 10^{-3}$	$5.42 \cdot 10^{-3}$	$3.34 \cdot 10^{-3}$
	<i>t</i> _{0.5}	NA	205.64	127.86	207.49
	<i>t</i> _{0.1}	NA	31.25	19.43	31.53
Ascorbic acid	<i>k</i>	NA	NA	$4.38 \cdot 10^{-3}$	$2.98 \cdot 10^{-3}$
	<i>t</i> _{0.5}	NA	NA	158.22	214.42
	<i>t</i> _{0.1}	NA	NA	24.04	35.34

k, the reaction rate constants (h^{-1}); *t*_{0.5}, the time; concentration will decrease about 50 % (h);
*t*_{0.1}, the time; concentration after which will about 10% (h); NA, not available.

variance (PC1) can be perceived as the mean sensitivity to degradation (average value of the drug during degradation). Small value of PC1 is connected with large values of all percentages (i.e., weak overall degradation), whereas large values correspond to large overall degradation. The second trend (PC2) is connected with differences in the shape of the curve. The small PC2 value indicates weak degradation in first 14 days, followed by deep degradation after this period. On the contrary, high PC2 is connected with rapid degradation during first days, but the last three time points did not show so quick further degradation.

Looking at the values of PCA scores (Figure 2B and 2C), one can notice that they occupy some curvilinear path in the reduced space. The curves with middle amount of overall degradation have low PC2 value, i.e., the degradation starts slowly. On the contrary, the curves with smallest and highest overall degradation have the PC2 value high. This means that these curves exhibit higher degradation in the beginning of investigated process. Looking at the PC scores marked against the investigated cephalosporins (Figure 2B), one can conclude that there is no strict clustering of the curves belonging to particular cephalosporins. A slight shift of cefepime (triangles) towards high PC1 values can be only observed. On the contrary, clear and visible trends can be observed when the scores are marked against the degradation agents (Figure 2C). The upper

right portion of the graph (high overall degradation and quick degradation start) is observed only for strong oxidizing reagents (hydrogen peroxide in both concentrations, potassium permanganate). The water samples (absence of degradation reagents) are located on the left “arm” of this V-shaped trend, together with iodine samples. The reductive agents (sodium thiosulfate and ascorbic acid) cause visible degradation to the drugs when comparing to water and iodine samples (moderately high PC1); however, they differ with PC2 value, as the start of degradation is in their case slow. Neglecting some rotation due to forced orthogonalization, one can conclude that the variance of PC2 is mainly between oxidizing and reductive reagents. An interesting conclusion can be drawn from Figure 2D, where the shift in the samples due to temperature increase (from 22 to 36°) is marked as arrow. It can be clearly seen that increase of the temperature makes extreme increase of degradation in the case of samples with the degradation lowest at all. When the degradation is moderate (right arm of the trend), the increase of overall degradation is not so large; however, the shift is always connected with increase of PC1 and going “along” the V-shaped path.

To further visualize the similarity of the reagents, the data set was rearranged as matrix containing 7 rows (reagents) and 56 columns (all distinct combinations of drug, time and temperature). Analyzing the results of hierarchical cluster analysis

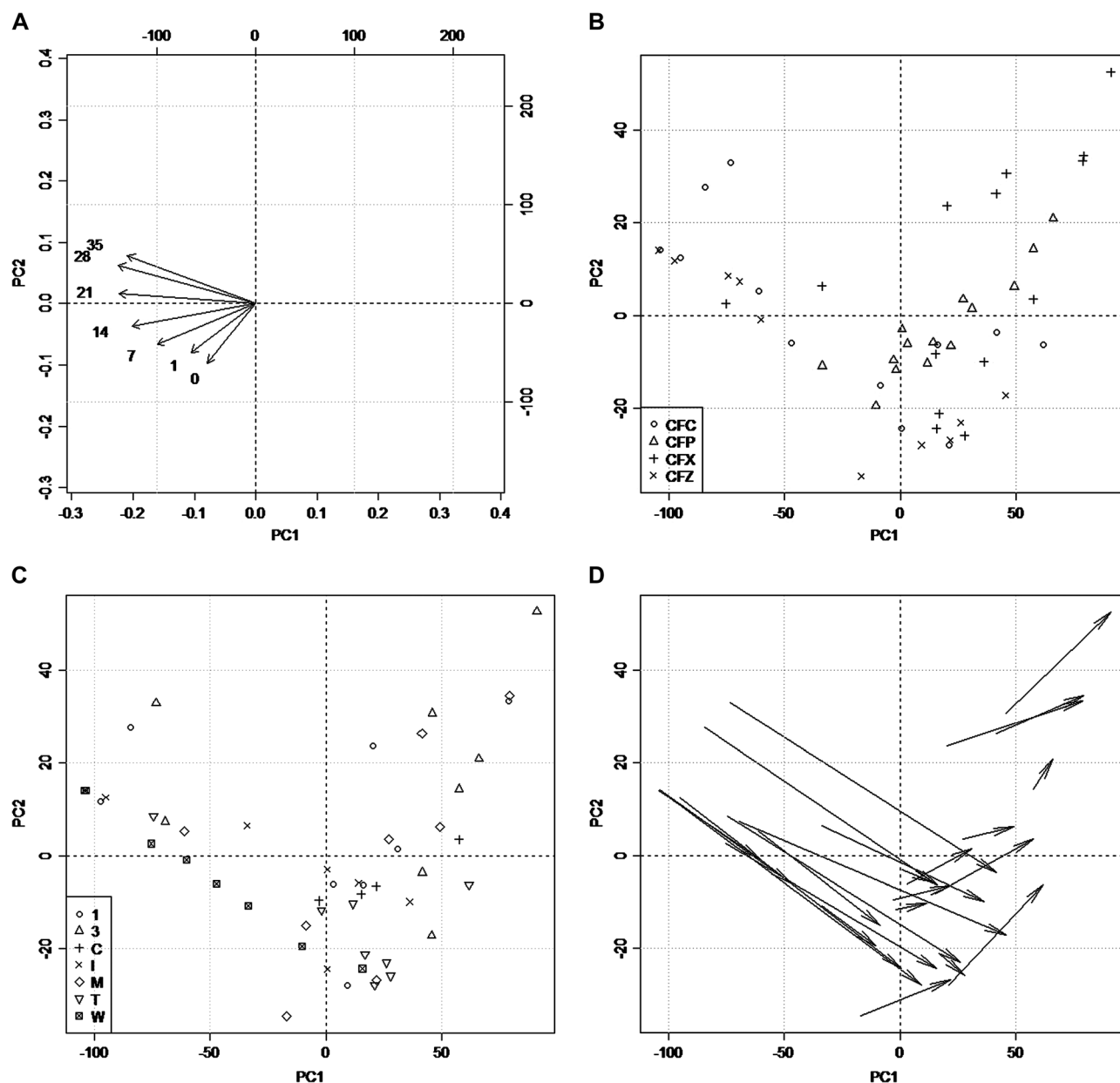


Figure 2. The loadings (A) and scores (B–D) of first two principal components of PCA analysis of degradation curves. (A) Curves are marked according to the drugs investigated; (B) curves are marked according to degradation agents. The movement in the reduced space between samples of temperature 22 and 36 °C are denoted as arrows in (C). (Abbreviations: 1 = 0.1% hydrogen peroxide, 3 = 0.3% hydrogen peroxide, C = ascorbic acid, I = iodine solution, M = potassium permanganate, T = sodium thiosulfate, W = water, CFC = cefaclor, CFP = cefepime, CFX = cefuroxime, CFZ = ceftazidime)

based on Euclidean distance (Figure 3), one can clearly see that these reagents cluster against their chemical mode of action. The most outlying cluster is water and iodine (which, surprisingly, is an oxidizer but does not act on investigated drugs). The agents causing degradation are visibly clustered to oxidative and reductive substances. The whole data set, arranged as a four-way array (tensor) of dimensions 7 (agents) \times 7 (time points) \times 4 (drugs) \times 2 (temperatures), was also subjected to parallel factor analysis. It was shown that one-factor model explains 92% of variance and is sufficient to model this data set. The scores of PARAFAC are shown in Figure 4.

As their values are modeled from percentages, the lower score value indicates stronger overall degradation. The analysis confirms the previous conclusion that the degradation in 36 °C is visibly stronger. The most stable drug is ceftazidime, whereas the less stable is cefuroxime axetil. The time points represent almost linear trend. Water and iodine have smallest degradation power, whereas potassium permanganate and 0.3% v/v hydrogen peroxide exhibit the strongest ability to degrade investigated drugs.

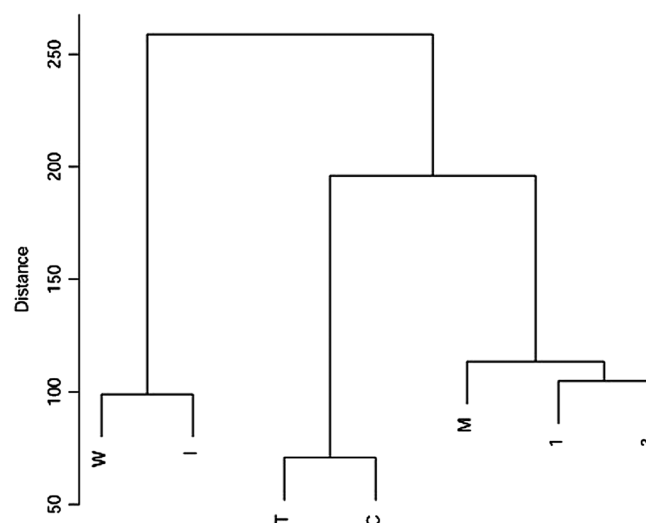


Figure 3. The Euclidean dendrogram showing the similarity between degradation agents. (Abbreviations as in Figure 2)

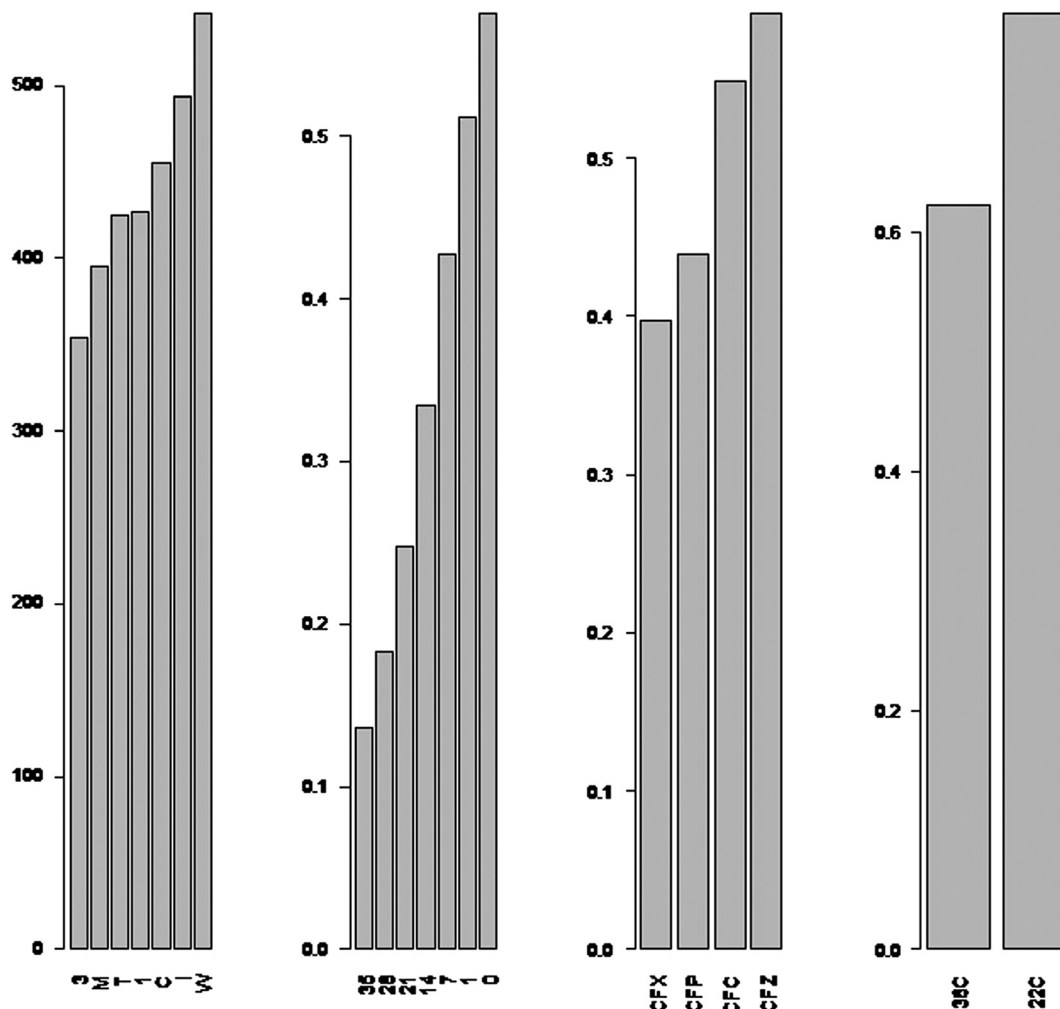


Figure 4. The scores of PARAFAC

Conclusions

The obtained parameters demonstrate that the increase of the temperature results in a lower stability of the drugs. The degradation rate of determined cephalosporins depends both on the temperature and the presence of the redox agents. The most stable drug is cefazolin, whereas the least stable is cefuroxime axetil. Water and iodine have smallest degradation power, whereas potassium permanganate and 0.3% hydrogen peroxide solutions exhibit the strongest ability to degrade the investigated drugs.

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